(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
8 November 2001 (08.11.2001)

PCT

(10) International Publication Number
WO 01/83816 A2

(51) International Patent Classification[7]: C12Q 1/68

(21) International Application Number: PCT/IB01/00899

(22) International Filing Date: 3 May 2001 (03.05.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/201,864 4 May 2000 (04.05.2000) US

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/83816 A2

(54) Title: METHOD FOR DETECTING TUMORS

(57) Abstract: Detection of tumors, including latent tumors, by analysing the bodily fluid of an individual, in particular by determining the presence or absence of tumors in a body fluid sample of mRNA encoding an extracellular matrix component in said sample, which component is specifically found in tumors. The component may be fibronectin ED-A, ED-B, or large isoform of tenascin C.

METHOD FOR DETECTING TUMORS

The present invention relates to a method for the detection of tumors, including occult tumors (i.e. tumors which are not detectable by standard procedures or careful examination using techniques such as CT scan or surgery). In particular the invention provides a method which is performed on bodily fluids, e.g., the peripheral blood of the individual.

The methods of the present invention are based on the detection of mRNA coding for a component of the extracellular matrix which accumulates around the neo-vasculature during the angiogenic processes which are involved in tumor formation. The methods use RT-PCR to detect the mRNA encoding said component. A preferred technique uses RT-PCR to detect mRNA containing the ED-B domain.

The present invention is based on the inventors' surprising discovery that mRNA encoding certain extracellular matrix components is actually present in the tumor cells themselves, and can be detected in the peripheral blood of patients with tumors, but not in healthy blood.

It is known that circulating tumor cells can be present in peripheral blood. This seems to be related to the presence of occult tumors, micro-metastasis and minimal residual disease in an individual. Their detection has therefore a diagnostic and prognostic value (Kvalheim (1998) Acta Oncol., 37, 455-462; Keilholz (1998) Eur. J. Cancer, 34, 837-841; Jung et al.(1998) British J. Cancer, 78, 1194-1198).

Monoclonal antibodies have been used to identify occult single cells derived from solid malignancies in the bone marrow and peripheral blood of many individuals (Moss and Sanders, (1990) J. Clin. Oncol. 8 736-740). However, this method has two main

limitations: poor sensitivity and false positivity due to cross-reaction of the monoclonal antibodies with normal cells.

Reverse transcriptase-polymerase chain reaction (RT-PCR)-based assays have also been used. These use tumor-specific expression of messenger RNA as a target for the characterization of tumor cells. Known RNA targets include the cytokeratins 18, 19 and 20 (CK18 CK19, CK20), tyrosinase, prostate-specific antigen (PSA), prostate-specific membrane antigen (PSMA), carcinoembrionic antigen (CEA), neuroendocrine protein gene product (PGP 9.5)(Kvalheim (1998) Acta Oncol., 37, 455-462).

However, known methods only allow a few specific kinds of tumors to be detected by the identification of the RNA targets (see Table 1).

RT-PCR is a highly sensitive technique, for example the detection of one tumor cell in more than 10^7 mononucleated cells has been achieved (Keilholz (1998) Eur. J. Cancer, 34, 837-841). A problem with RT-PCR based techniques is that the high sensitivity means that it is difficult to discriminate between positive and negative samples. There could be background detection leading to false positives results, for example due to the presence of genomic DNA in the samples, or due to the presence in occasional cells of an mRNA with the same or similar sequence.

The present invention provides a method for the broad diagnosis of malignancies and residual disease by assaying the bodily fluids to detect an mRNA which is found in a broad range of tumor cells.

The present invention is based on the inventors' experimental work using RT-PCR to detect the presence of the ED-B and ED-A

domains of fibronectin, and tenascin-C large isoform. These represent pan-tumor markers, since their presence in extracellular matrix associated with a tumor is not constrained to a particular type of tumor.

Tumors cannot grow beyond a certain mass without the formation of new blood vessels (angiogenesis), and a correlation between microvessel density and tumor invasiveness has been reported for a number of tumors (Folkman, Nat. Med. 1: 27, 1995). The ED-B domain of fibronectin, a sequence of 91 amino acids, which is inserted by alternative splicing into the fibronectin molecule, specifically accumulates around neovascular structures(Zardi et al. (1987) EMBO J, 6, 2337-2342; Carnemolla et al. (1992) J. Cell Biol., 108, 1139-1148).

The domain is usually absent from normal adult tissues (Zardi et al. (1987) EMBO J, 6, 2337-2342; Carnemolla et al. (1996) Int. J. Cancer 68 397-405; Tarli et al. (1999) Blood 94 192-198; Castellani et al. (1994) Int. J. Cancer 59 612-618). There have also been reports that some specific types of tumor might express ED-B-containing fibronectin (Tavian et al. (1994) Int. J. Cancer, 56, 820-825); Carnemolla et al., (1989) J. Cell Biol,108 1139-1148; Mariani et al. (1997) Cancer, 80, 2378-2384; Pujuguet et al.,(1996) Am. J. Pathol. 148 579-592).

Specifically, the invention is based in various aspects upon the inventors' surprising finding that mRNA containing the ED-B domain is found in the vast majority of tumor cells themselves and is not limited, as previously thought, to the neovasculature associated with the tumors. They show that tumor cells can be detected by RT-PCR of the ED-B+ mRNA, when diluted in blood. The inventors also show that mRNA containing the ED-B domain can be detected in the peripheral blood of tumor bearing animals, but, importantly for discrimination, not healthy animals.

The inventors also show that the mRNA of extracellular domains ED-A, tenascin C large isoform (particularly the repeat D) can be detected in transformed cells but not in the blood of healthy donors.

It is surprising that RT-PCR directed at extracellular matrix mRNA is able to discriminate between the blood of healthy individuals and the blood of individuals with tumors. This is particularly true for Aonco-fetal markers@ of which ED-B is one.

The present invention provides a method of determining the presence or absence of tumors in an individual, comprising: performing reverse transcription PCR (RT-PCR)on a sample of bodily fluid taken from an individual to determine the presence or absence of a messenger RNA encoding an extracellular matrix component, which mRNA is present in tumors, said RT-PCR reaction comprising the use of at least one primer specific for said mRNA, thereby determining the presence or absence of tumors in the individual.

Before performing the RT-PCR, the sample may be treated, e.g. concentrated, by methods known in the art.

It is preferred that the extracellular matrix component is selected from the group: ED-B domain of fibronectin; the large isoform of tenascin C; repeat C of tenascin C; repeat D of tenascin-C; or the ED-A domain of fibronectin.

The bodily fluid may be blood, plasma, serum, cerebro-spinal fluid, needle aspirates, tissue samples, tumor samples, saliva and urine. Preferably the bodily fluid is blood, e.g., peripheral blood.

The primers used in the PCR reaction are specific for the extracellular matrix component mRNA which is present in tumors. Where the extracellular matrix component is present in tumors as a result of splicing, it is preferred that at least one of the primers overlaps either the 5' or the 3' splice junction, preferably a first PCR primer overlaps the 5' junction and a second PCR primer overlaps the 3' splice junction. In this situation the forward primer may comprise sequence of the 5' extremity of the tumor specific exon and sequence of the 3' extremity of the exon which is 5' to the tumor specific exon. The reverse primer may comprise sequence of the 3' extremity of the tumor specific exon and sequence of the 5' extremity of the exon which is 3' to the tumor specific exon.

In a preferred embodiment the mRNA is the fibronectin ED-B domain mRNA. A preferred forward primer comprises sequence of the 5' extremity of the ED-B exon and sequence of the 3' extremity of the fibronectin repeat 7 (i.e. overlaps the 5' splice junction). A preferred reverse primer comprises sequence of the 3' extremity of the ED-B exon and sequence of the 5' extremity of the fibronectin repeat 8 (i.e. overlaps the 3' splice junction). The preferred PCR primers are FN7-EDB and EDB-FN8 described herein. In accordance with the requirements of PCR, the reverse primer EDB-FN8 has the same sequence as the target cDNA and the forward primer FN7-EDB is complementary to the target cDNA.

RT-PCR is well understood by the person skilled in the art. A typical procedure may include:
contacting said sample with a first nucleic acid primer hybridisable to an mRNA encoding an ED-B domain of fibronectin;
synthesising a DNA molecule complementary to said mRNA;
mixing said complementary DNA with at least a second nucleic

acid primer hybridisable to said DNA;
performing a polymerase chain reaction on said DNA;
and analysing the PCR product to determine the presence or absence in said sample of an mRNA encoding said ED-B domain, by analysis of the PCR product.

The PCR product may be analysed by a technique known in the art, for example by examining the size of the product, by restriction digest of the product, probing the product, or sequence analysis of the product. Exemplary methods are discussed further below.

As an alternative to PCR, other functionally equivalent nucleic acid amplification or detection techniques may also be used (see for example Nolte, (1998) Adv. Clin. Chem. 33 201-235

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows alternative pre-mRNA splicing of the ED-B exon in normal and tumoral cells. In the fibronectin (FN) gene and FN primary transcript, two introns separate ED-B exon from the sequences coding for domains 7 and 8 of fibronectin. In normal cells (such as blood leukocytes), the exon of ED-B is removed together with the adjacent introns during pre-mRNA splicing. Therefore, the resulting mRNA is devoid of ED-B exon and the sequences coding for repeat 7 and repeat 8 are joined together.

However, the splicing machinery of tumoral cells does not remove ED-B exon from the primary transcript but only the flanking introns. In the resulting mRNA, the exons coding for repeat 7, ED-B and repeat 8 of fibronectin follow each other.

Figure 2 shows the primers used for the selective

amplification of the ED-B exon from cDNA and a schematic description of the amplification of the ED-B exon from cDNA, but not from genomic DNA or primary transcript, which can be obtained by RT-PCR.

The forward primer overlaps the 5' extremity of ED-B exon and part of the 3' extremity of the exon coding for the adjacent repeat 7 of FN. The reverse primer overlaps the 3' end of ED-B exon and the 5' extremity of the exon coding for the adjacent repeat 8 of fibronectin. Only in mRNA of tumoral cells, where the exons are adjacent, the primers find the correct annealing sequence.

A. Methods

The methods make use of bodily fluid samples from individuals which may for example, have or be suspected of having tumors. Examples of bodily fluids include blood, plasma, serum, tissue samples, tumor samples, saliva and urine, blood is the preferred bodily fluid, in particular peripheral blood. Comparison may be made with samples taken from normal individuals, or with samples previously taken from an individual under scrutiny.

mRNA is detected using RT-PCR. In RT-PCR, a cDNA is first transcribed from the mRNA. The primer used to initiate transcription may be specific for the mRNA of interest, or could be hybridisable to the polyA tail of mRNA, i.e. could be an "oligo dT" primer, as is understood by the person skilled in the art. PCR is then performed on the resultant cDNA. At least one additional primer is used in the PCR reaction, which is complementary to the mRNA of interest. In this case the primer used to prime the cDNA synthesis acts as the second PCR primer. Preferably at least two primers are added in the PCR reaction, one primer being the forward primer to the cDNA of

interest and one being the reverse primer, e.g. this could be specific for the mRNA of interest, or could be an "oligodT"-based primer. It is preferred that both primers in the PCR reaction are specific for the cDNA of interest. Where the mRNA is present in tumors as a result of a splicing event, it is preferred that at least one of the primers overlaps a splice junction as discussed elsewhere herein.

PCR techniques for the amplification of nucleic acid are described in US Patent No. 4,683,195. In general, such techniques require that sequence information from the ends of the target sequence is known to allow suitable forward and reverse oligonucleotide primers to be designed to be identical or similar to the polynucleotide sequence that is the target for the amplification. References for the general use of PCR techniques include Mullis et al, Cold Spring Harbor Symp. Quant. Biol., 51:263, (1987), Ehrlich (ed), PCR technology, Stockton Press, NY, 1989, Ehrlich et al, Science, 252:1643-1650, (1991), "PCR protocols; A Guide to Methods and Applications", Eds. Innis et al, Academic Press, New York, (1990).

On the basis of amino acid sequence information, oligonucleotide probes or primers may be designed, taking into account the degeneracy of the genetic code, and, where appropriate, codon usage of the organism from the candidate nucleic acid is derived. An oligonucleotide for use in nucleic acid amplification may have about 10 or fewer codons (e.g. 6, 7 or 8), i.e. be about 30 or fewer nucleotides in length (e.g. 18, 21 or 24). Generally, specific primers are upwards of 14 nucleotides in length, but not more than 18-20. Those skilled in the art are well versed in the design of primers for use processes such as PCR.

The amplified nucleic acid (PCR product) may then be

sequenced, and/or tested in any other way to determine the presence or absence of a particular sequence, for example by determining the size of the PCR product on an agarose gel, by probing the product with a suitably labelled probe specific for the sequence of interest, or by restriction enzyme digest and electrophoresis.

Sequencing of a PCR product may involve precipitation with isopropanol, resuspension and sequencing using a TaqFS+ Dye terminator sequencing kit. Extension products may be electrophoresed on an ABI 377 DNA sequencer and data analysed using Sequence Navigator software.

A variant form of the mRNA may contain one or more insertions, deletions, substitutions and/or additions of one or more nucleotides compared with the wild-type sequences. Differences at the nucleic acid level are not necessarily reflected by a difference in the amino acid sequence of the encoded polypeptide.

Nucleic acid according to the present invention, such as a full-length coding sequence or oligonucleotide probe or primer, may be provided as part of a kit, e.g. in a suitable container such as a vial in which the contents are protected from the external environment. The kit may include instructions for use of the nucleic acid, e.g. in PCR and/or a method for determining the presence of nucleic acid of interest in a test sample. A kit wherein the nucleic acid is intended for use in PCR may include one or more other reagents required for the reaction, such as polymerase, nucleosides, buffer solution etc. The nucleic acid may be labelled. A kit for use in determining the presence or absence of nucleic acid of interest may include one or more articles and/or reagents for performance of the method, such as means for providing the test sample itself, e.g. a syringe for removing

a blood sample (such components generally being sterile).

B. EXTRACELLULAR MATRIX COMPONENTS

The mRNA coding for the extracellular matrix domain which is the target of the PCR reaction is one which is present in tumors. It is preferred that the mRNA is one which is expressed to an enhanced level in tumor cells compared with normal adult cells. For example, the extracellular domain may be spliced out in adult cells but spliced into mRNA in tumor cells. Examples of suitable mRNAs include fibronectin ED-B domain; fibronectin ED-A domain, tenascin C large isoform; repeat D of tenascin C large isoform; MUC1 (Hartman et al. (1999) Int. J. Cancer 82 256-267); CD 44 (Naot et al. (1997) Adv. Cancer Res. 71 241-319; Li et al. BJU Int. 85 514-518); Versican (Paulus et al. (1996) J. Neuropathol. Exp. Neurol. 55 528-533), IIICS domain of fibronectin (Zardi et al. (1987) EMBO J, 6, 2337-2342).

The preferred extracellular matrix domain is the fibronectin ED-B domain. In this case the mRNA encoding the domain is spliced out in almost all normal adult tissues. Other examples include the large isoform of Repeat D of tenascin C, and the ED-A domain of fibronectin, as discussed further below.

Accordingly, other sequences which code for components of the modified extracellular matrix, and which are inserted or omitted by alternative splicing of the primary transcript and could also be used for the detection of tumor cells in blood.

For example, the insertion (by a mechanism of alternative splicing) of the ED-A domain into fibronectin, and/or of a set of domains into the tenascin-C molecule, is associated to pathological conditions, including malignancies (Castellani et al., (1986) J. Cell Biol. 104 595-600; Borsi et al.(1987) J.

Cell Biol. 104 595-600; Carnemolla et al. (1999) Am. J. Pathol. 154 1345-1352). The Alarge@ tenascin isoforms (which include a number of extra domains inserted by alternative splicing) are abundant components of the extracellular matrix of many tumors, such as breast and brain tumors (Borsi et al. (1992) Int. J. Cancer 52 688-692), but are undetectable in many normal tissues. In particular, the extra-domain C of tenascin-C is undetectable in virtually all normal adult tissues, but extremely abundant in high grade astrocytoma, especially around vascular structures and proliferating cells (Carnemolla, 1999). Other domains of interest include MUC1, CD 44, Versican, and the IIICS domain of fibronectin as described above.

The invention also encompasses the development of a multiple marker RT-PCR test, based on the detection of the insertion of domains into components of the modified extracellular matrix. This should further increase the reliability and sensitivity of tumor cell detection in blood, and possibly other body fluids (Sauter et al.(1999) British J. Cancer 81 1222-1227; Nakanishi et al. (1999) Cancer Chemother Pharmacol. 43 (Suppl.) S32-S36).

The invention will now be illustrated in detail with reference to the following examples.

<u>EXAMPLE 1: ED-B+ mRNA CAN BE DETECTED BY RT-PCR IN TUMOR AND TRANSFORMED CELL LINES.</u>

RT-PCR using primers specific for the ED-B mRNA was performed on total RNA isolated from 9 different tumoral cell lines.

We produced RT-PCR oligonucleotide primers which anneal to ED-B only when it is inserted in mRNA. These primers therefore avoid false positives due to amplification of genomic DNA or

primary transcript.

As shown in Figure 2, the forward primer overlaps the 5′ extremity of the ED-B exon and part of the 3′ extremity of the exon coding for repeat 7 of fibronectin. The reverse primer overlaps the 3′ extremity of ED-B exon and the 5′ extremity of the exon coding for repeat 8 of fibronectin. Since the exons ED-B/FN7 and the exons EDB/FN8 are separated by an intron in genomic DNA and in the primary transcript these primers will anneal only to in mRNA, because it has undergone alternative splicing.

Amplification of the ED-B exon from cDNA but not from genomic DNA or primary transcript, can be obtained by RT-PCR performed with the primers schematically represented in Figure 1.2. The forward primer overlaps the 5′ extremity of ED-B exon and part of the 3′ extremity of the exon coding for repeat 7 of FN. The reverse primer overlaps the 3′ end of ED-B exon and the 5′ extremity of the exon coding for repeat 8 of fibronectin. The primers only find the correct annealing sequence in those cells where the exons are adjacent.

For all the tested cell lines and tumors, amplification of ED-B exon was clearly detectable on agarose gel. ED-B+mRNA seems therefore a suitable pan-tumor marker for RT-PCR based tumor diagnosis.

<u>Material and Methods</u>

Cell lines

The SV40-transformed WI-38-VA cell line, the F9 murine teratocarcinoma (Tarli et al. 1999), the FE-8 ras-transformed rat fibroblasts, the C-51 murine colon carcinoma (Tarli et al., 1999), the SK-MEL28 human melanoma, and the LOVO

colorectal cancer cell line, were grown in Dulbecco Modified Eagle's Medium (DMEM), 10% Fetal Calf Serum (FCS).

The EA.hy 926 human lung cancer cell line, the C6 rat glial tumor cells, and the T-24 human bladder carcinoma cells were cultured respectively in: Dulbecco's HAT medium, 10% FCS; H199 medium, 10% FCS; McCoy's medium 10%FCS.

All cell lines described herein were kindly provided by Prof. H. Wunderli-Allenspach.

RNA extraction

Cultured cells were dissociated with trypsin and total RNA isolated using the Rneasy Mini Kit (QIAGEN, Basel, Switzerland).
Concentration and purity of RNA was determined by measuring the absorbance at 260 nm and 280 nm in a spectrophotometer (an adsorbance of 1 unit at 260 nm corresponds to a RNA concentration of 40 µg/ml whereas the ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity). Quality of total RNA was checked by formaldehyde-agarose gel electrophoresis and ethidium bromide staining.

RT-PCR

RT-PCR was performed with the two-enzymes Access RT-PCR System (Promega), following the manufacturer's instructions. 100pg-1µg of total RNA were used as template. For the detection of ED-B+ mRNA, the primers FN7-EDB (5′ tct gat acc atc atc cca gag gtg ccc 3′ - SEQ ID NO. 1) and EDB-FN8 (5′ ggg agg agg aac agc cgt ttg ttg 3′ - SEQ ID NO. 2) were used. As control, RT-PCR with primers specific for the housekeeping gene β-microglobulin, was performed.
RT-PCR reactions were performed on a thermal cycler running

the program: 48°C(45′)-[94°C(2′)-60°C(1′)-68°C(2′)]$_{40cycles}$-68°C(7′).

RT-PCR products were analyzed by electrophoresis on 1.4% agarose gel.

Results

RT-PCR with primers FN7-EDB and EDB-FN8 was performed on RNA isolated from different cell lines: WI-38-VA human transformed fibroblasts, F9 murine teratocarcinoma cells, FE-8 cells, FE-8 ras-transformed rat fibroblasts, C-51 murine colon cacinoma, SK-MEL28 human melanoma, LOVO colorectal cancer cell line, T24 human bladder carcinoma, C6 rat glial tumor cells, EA.hy 926 human lung cancer cell line. The PCR products were run on an agarose gel. For all the tested cell lines, amplification of the ED-B exon from the mRNA was detected as a band of 280 bp. All the tested cell lines contain a detectable amount of mRNA which is positive for the presence of ED-B exon.

This shows that tumor and transformed cell lines of different origin produce detectable amounts of ED-B+ mRNA. The amplification products did not derive from amplification of ED-B exon from genomic DNA, since we did not obtain any amplification when reverse transcriptase was omitted from the reactions.

As a control, RT-PCR performed with primers β-micro-f and β-micro-r, gave amplification of the housekeeping gene β-microglobulin, confirming that the isolated RNA could be retrotranscribed and further amplified.

EXAMPLE 2:ED-B+ mRNA CANNOT BE DETECTED BY RT-PCR IN PERIPHERAL BLOOD FROM HEALTHY DONORS UNLESS TUMORAL CELLS ARE PRESENT IN THE BLOOD

We here show that blood cells of three human healthy donors do not contain B+mRNA detectable by RT-PCR. Then, the situation where an individual has disseminated tumor cells in peripheral blood was simulated by adding F9 cells to the peripheral blood obtained from an healthy donor. 10^4 F9 cells in 10^6 blood cells allowed the detection of ED-B+mRNA by RT-PCR, the intensity of the signal increasing proportionally with the number of WI-38-VA cells added to the blood cells.

Material and Methods

Peripheral blood

Whole peripheral blood from healthy human donors, collected in EDTA, was obtained by the "Blutspendendienst of Zürich". Blood was processed within a maximum of 4 hours after collection.

RNA extraction

Total RNA from fresh whole human peripheral blood was isolated using the QIAamp RNA Blood Mini Kit (QIAGEN, Basel, Switzerland). The same kit was used for the isolation of RNA from blood added with cultured WI-38-VA cells. Concentration, purity, quality of the isolated RNA was determined as reported in Example 1.

RT-PCR

RT-PCR reactions were performed as reported in Example 1.

Results

We here show that ED-B+mRNA cannot be detected by RT-PCR in blood cells from healthy donors, and that tumor cells can be

detected by RT-PCR of the ED-B+ mRNA, when diluted in blood.

Peripheral blood was obtained from human healthy donors. In 1 ml of blood sample, different amounts of F9 cells, ranging from 0 to 10^5, were diluted. RNA was isolated from the different samples and RT-PCR performed with primers FN7-EDB and EDB-FN8. The products were analysed on agarose gels. ED-B+mRNA was undetectable in the samples with no F9 cells, 10^2 WI-38-VA cells and 10^3 WI-38-VA cells were added to 1 ml of blood. When 10^4 WI-38-VA cells were added to the blood ED-B+mRNA was detectable as a weak amplification signal. The intensity of the signal increased proportionally with the increase of the number of WI38VA cells diluted into the blood.

As control, we performed RT-PCR with primers β-micro-f and ß-micro-r, which gave amplification of the β2-microglobulin gene, and we omitted reverse transcriptase from the RT-PCR tests obtaining no amplification signal.

<u>EXAMPLE 3:</u> <u>ED-B+ mRNA can be detected in peripheral blood from tumor bearing mice but not in peripheral blood of healthy mice.</u>

In the previous examples, we showed that ED-B+mRNA is not detectable by RT-PCR in peripheral blood cells from healthy donors. However, when tumor cells are diluted in this blood sample, their presence was identified by RT-PCR detecting ED-B+mRNA.

In this example we have detected circulating tumor cells in a relevant <u>in vivo</u> model. We subcutaneously implanted a FE-8 rat sarcoma in nude mice. Once the tumor size reached 500 mm^3, we removed 1 ml of peripheral blood per mouse and we isolated the total RNA from blood. ED-B+mRNA was detected by the RT-PCR approach described in this application.

In contrast, no detection of ED-B+mRNA was possible, when RT-PCR was performed on peripheral blood isolated from healthy mice.

Materials and Methods

Mouse model

Nude mice (12-week-old Swiss nudes, females) were subcutaneously implanted with FE-8 rat carcinoma cells. When tumor size was approx. 500 mm^3 (tumors are measured with a dial-caliper and the volume determined using the formula: $width^2$ x Length x 0.52), 1 ml of peripheral blood was extracted from each mouse.

RNA extraction

Extraction of RNA and determination of its purity, concentration and quality was performed as reported in Example 2.

RT-PCR

RT-PCR was performed as described in Example 1, using primers FN7-EDB and EDB-FN8.

Results

Tumoral cells circulating in peripheral blood were identified in a murine model by RT-PCR detecting ED-B+mRNA.

We performed RT-PCR with primers FN7-ED-B and EDB-FN8 on RNA isolated from peripheral blood of 5 tumor bearing mice and 5

healthy mice. The tumor chosen for the experiment is the FE-8 rat sarcoma, which is positive for the presence of ED-B+mRNA (see Example 1).

Peripheral blood was obtained either from healthy mice or from mice bearing a subcutaneously grafted FE-8 rat sarcoma. RNA was isolated from the blood sample and RT-PCR performed with primers FN7-EDB and EDB-FN8. The PCR products were analysed on agarose gels. EDB+ mRNA was detected in blood from tumor bearing mice but not from blood of healthy mice. This showed that for blood extracted from healthy mice, no amplification product is detectable on agarose gel. In contrast, amplification of the ED-B exon from mRNA was detectable when blood had been taken from tumor-bearing mice.

As a control, we performed RT-PCR with primers specific for the housekeeping gene ß-microglobulin, and we omitted reverse transcriptase from the RT-PCR tests, obtaining no amplification signal.

<u>EXAMPLE 4: mRNA CONTAINING THE SEQUENCE CODING FOR REPEAT D OF TENASCIN-C AND FOR EXTRA DOMAIN A OF FIBRONECTIN CAN BE DETECTED BY RT-PCR IN WI-38-VA CELLS, BUT NOT IN BLOOD CELLS FROM HEALTHY DONORS.</u>

Other examples, in which a mechanism of pre-mRNA alternative splicing gives rise to protein isoforms differentially expressed in normal and tumoral tissues. One is the Extra Domain A (ED-A) of fibronectin. There is an increased expression of fibronectin isoforms containing ED-A associated with transformed cells and malignancies, compared to normal cells and tissues (Castellani et al.(1986) J. Cell Biol. 103 1671-1677). Tenascin-C is another extracellular protein which exists in several polymorphic isoforms due to alternative splicing of nine fibronectin-like type III repeats. Large

Tenascin-C isoforms are present in some normal adult tissues but are upregulated in neoplastic tissues (Borsi et al., 1992). Most large tenascin-C isoforms contain the extra domain D (Borsi et al., 1992).

We used a similar RT-PCR approach designed for the detection of ED-B+mRNA, to test if tumor cells contain ED-A+ and TNlarge messenger RNA (particularly, the repeat D of tenascin-C). The presence of ED-A+ and TNlarge mRNA in a human transformed cell line, indicates that also these mRNAs form are suitable markers for the detection of tumoral cells. Possibly, a combined RT-PCR test which detects several alternatively spliced exons of mosaic proteins of the extracellular matrix (e.g., the ED-B domain of FN, the ED-A domain of FN, the IIICS domain of FN, the D domain of TN-C, the C domain of TN-C) may provide a more informative identification of circulating cells in the blood of individuals with cancerous lesions.

Materials and Methods

Cell line

The SV-40 transformed WI-38-VA cell line was grown as reported in Example 1.

RNA extraction

Extraction of RNA and determination of its purity, concentration and quality was performed as reported in Example 1.

RT-PCR

RT-PCR was performed as described in Example 1. For the detection of ED-A+mRNA the primers FN11-EDA (5′ gtt cag act

gca gta acc gac att gat 3' - SEQ ID NO. 3) and EDA-FN12 (5' tgg tgc agg aat agc tgt gga ctg 3' - SEQ ID NO. 4) were used. For the detection of largeTN-C mRNA, containing the repeat D, primers TNDba (5' agc cga acc gga agt tga ca 3' [redacted] and TN6D (5' ttt ggg gag ccc atg gct gtt gtt gct 3' - SEQ ID NO. 6) were synthesised.

Results

Total RNA was isolated from 10^6 WI-38-VA cells and RT-PCR performed with primers FN11-EDA and EDA-FN12 or with primers TNDba and TN6D. Amplification of the ED-A exon was obtained, as well as amplification of the exon coding for domain D of TN-C, whereas the result was negative when Reverse Transcriptase was omitted from the test and when the test was performed on blood from healthy donors. Furthermore, similar RT-PCR reactions performed with normal human blood did not amplify the ED-A and the repeat D exons.

The oligonucleotide primer TNDba, for amplification of the repeat D from the tenascin trascript, does not discriminate between transcripts containing or devoid of the adjacent repeat C, which undergoes additional splicing control (Carnemolla et al., 1999). Choosing other backward primers would allow the discrimination betweeen tenascin transcripts containing the repeat D, in the presence or the absence of the adjacent repeat C. Such investigations would be of value, since the repeat C of tenascin-C is undetectable in virtually all normal adult tissues, but extremely abundant in aggressive tumors such as high grade astrocytoma, especially around vascular structures and proliferating cells (Carnemolla et al. 1999)

For nomenclature of the tenascin-C transcripts, see Carnemolla et al. (1999). RT-PCR of normal human blood is negative.

This shows that tumor cells contain detectable amounts of both ED-A+ mRNA and large form of TN-C mRNA. We used the RT-PCR approach designed for the detection of ED-B+ mRNA in tumor cells. For the detection of ED-A+mRNA we performed RT-PCR with primers annealing to ED-A exon and to the exon coding for the flanking FN repeats (the forward primer overlaps the 5′ extremity of ED-A exon and the 3′ extremity of the exon coding for the adjacent repeat 11; the reverse primer overlaps the 3′ end of ED-A exon and the 5′ extremity of the adjacent exon coding for repeat 12 of FN). For the detection of the large form of TN-C mRNA, we designed a forward primer [oligo TN6D], which anneals to the exon coding for repeat D of TN-C and to the the following exon (which codes for a repeat which is not subjected to alternative splicing). We also used a backward primer [oligo TNDba], which anneals only to the D repeat of TN-C.

Tumor origin	**Marker**
Pancreas	Carcinoembryonic Antigen (CEA) Ki-ras
Prostate	Prostate Specific Antigen (PSA), Prostate Specific Membrane Antigen (PSM)
Melanoma	Tyrosinase
Neuroblastoma	Neuroendocrine Proteing Gene Product 9.5 (PGP 9.5)
Breast Cancer	Cytokeratin 19 (CK19)

Table 1: Detection of occult solid cancer cells in blood by

reverse-transcriptase polymerase chain reaction (RT-PCR)(adapted from Kvalheim 1998)

SEQUENCES

SEQ ID NO. 1

tct gat acc atc atc cca gag gtg ccc

SEQ ID NO. 2

ggg agg agg aac agc cgt ttg ttg

SEQ ID NO. 3

gtt cag act gca gta acc gac att gat

SEQ ID NO. 4

tgg tgc agg aat agc tgt gga ctg

SEQ ID NO. 5

agc cga acc gga agt tga ca

SEQ ID NO. 6

ttt ggg gag ccc atg gct gtt gtt gct

CLAIMS:

1. A method for determining the presence or absence of tumors in an individual, the method comprising:

performing nucleic acid amplification on a sample of bodily fluid taken from an individual to determine the presence or absence of a messenger RNA encoding an extracellular matrix component, which mRNA is present in tumor cells, said nucleic acid amplification employing at least one primer specific for said mRNA, thereby determining the presence or absence of tumors in the individual.

2. A method according to claim 1 wherein the bodily fluid is blood.

3. A method according to claim 1 wherein the nucleic acid amplification involves RT-PCR.

4. A method according to any one of claims 1 to 3 wherein the extracellular matrix component is selected from the group of: the ED-B domain of fibronectin; the ED-A domain of fibronectin; the large isoform of tensacin C; the repeat C of tenascin C; the repeat D of tenascin C.

5. A method according to claim 4 wherein the extracellular matrix domain is the ED-B domain of fibronectin.

6. A method according to claim 4 wherein the extracellular matrix domain is the large isoform of tenascin C.

7. A method according to claim 4 wherein the extracellular matrix domain is the repeat C of tenascin C.

8. A method according to claim 4 wherein the extracellular matrix component is the repeat D of tenascin C.

9. A method according to claim 4 wherein the extracellular matrix domain is the ED-A domain of fibronectin.

10. A method according to claim 1 wherein the extracellular matrix component is the product of a splicing reaction and wherein RT-PCR is performed using at least one primer which overlaps a splice-junction of said extracellular matrix component.

11. A method according to any one of claims 1 to 10 wherein the presence of said messenger RNA is quantitated.

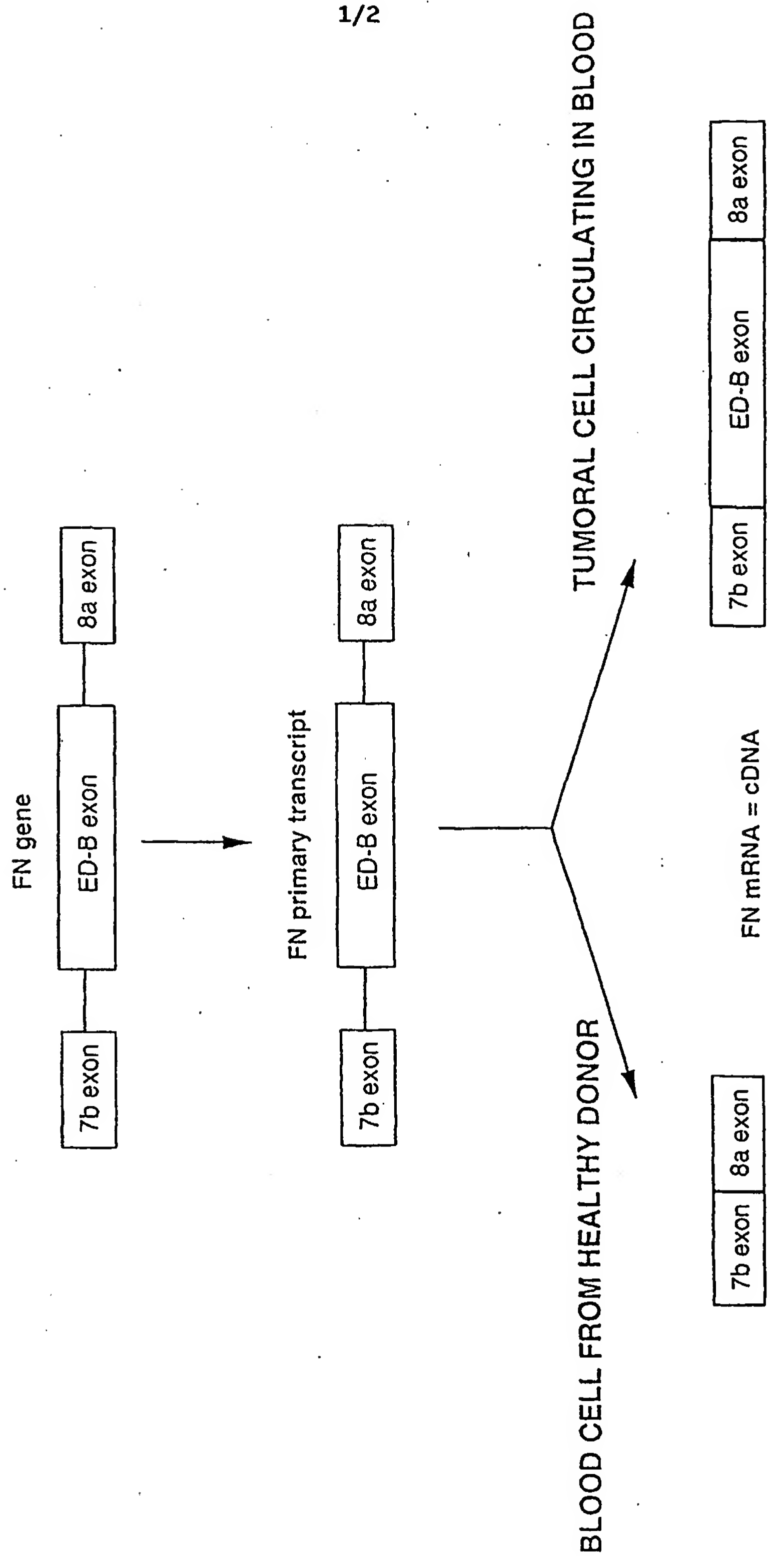
FN gene
7b exon
ED-B exon
8a exon
FN primary transcript
7b exon
ED-B exon
8a exon
BLOOD CELL FROM HEALTHY DONOR
TUMORAL CELL CIRCULATING IN BLOOD
7b exon
8a exon
7b exon
ED-B exon
8a exon
FN mRNA = cDNA

Figure 1

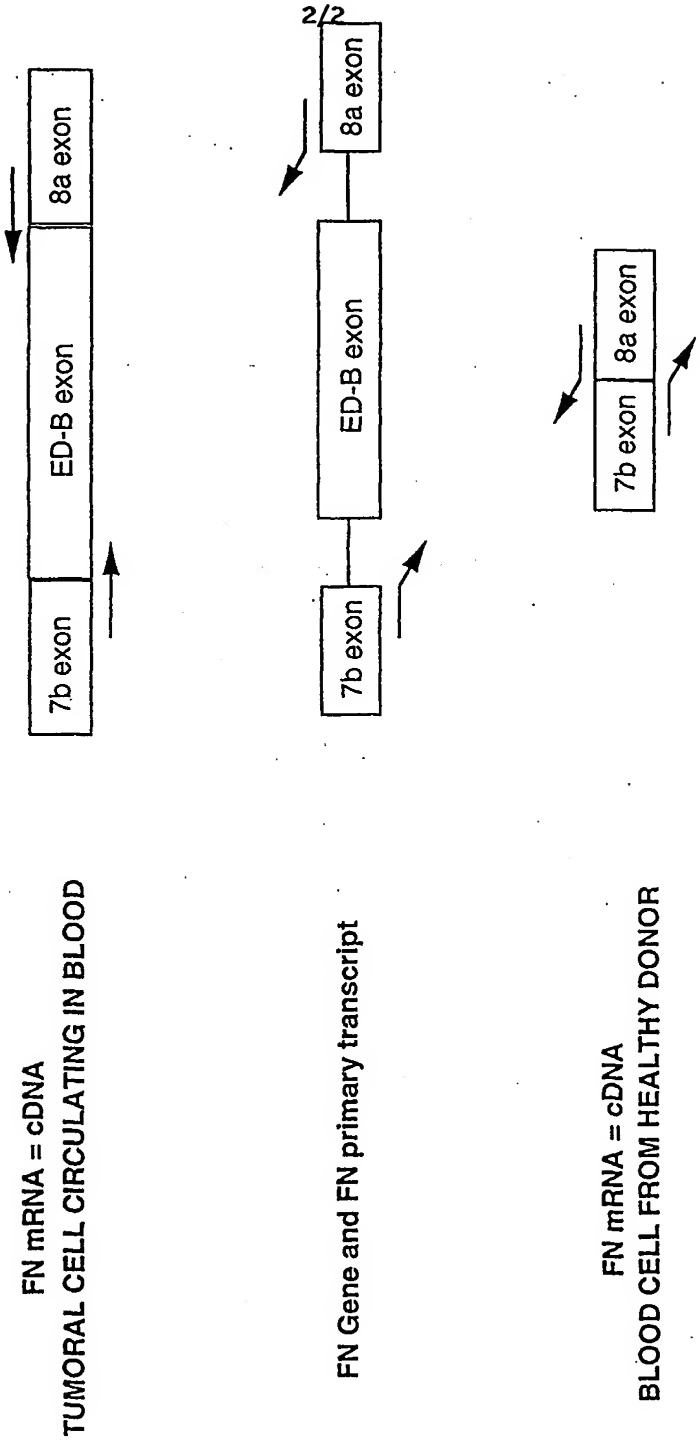
7b exon
ED-B exon
8a exon
FN mRNA = cDNA
TUMORAL CELL CIRCULATING IN BLOOD
7b exon
ED-B exon
8a exon
FN Gene and FN primary transcript
7b exon
8a exon
FN mRNA = cDNA
BLOOD CELL FROM HEALTHY DONOR

Figure 2